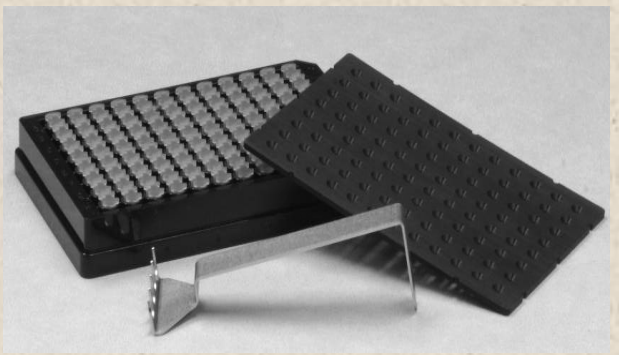
Cell Migration Assay Kit*

96-well, 2-D Assay for Investigating
Cell Migration of Adherent Cell Lines

PROTOCOL & Instructions for use

* Patent Pending



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Bringing Science to the Surface

I. INTRODUCTION

The Oris™ Cell Migration Assay is a reproducible, sensitive, and flexible assay that can be used to monitor cell migration. Formatted for a 96-well plate, the assay utilizes Oris™ Cell Seeding Stoppers (made from a medical-grade silicone) to restrict cell seeding to the outer annular regions of the wells. Removal of the stoppers reveals a 2mm diameter unseeded region in the center of each well, i.e., the migration zone, into which the seeded cells may then migrate. The Oris™ Migration Mask is applied to the plate bottom and restricts visualization to the migration zones, thus allowing only migrated cells to be detected (see Figure 1). The Oris™ Cell Migration Assay is designed to be used with any commercially available stain or labeling technique and the readout can be performed by microscopic examination or by using a plate reader.

The Oris™ Cell Migration Assay system has been designed for use with adherent cell cultures. This assay has been successfully used with fibroblast (NIH-3T3), fibrosarcoma (HT1080), and endothelial (HCEC and MCF10A) cell lines.

Using the Oris™ Cell Migration Assay offers the following benefits:

- **Membrane-free Migration** - no transwell inserts to manipulate
- **Reproducible Results** - the unique design provides well-to-well CV's < 12%
- **Preserves Cell Morphology** - changes in cell structure can be monitored in real-time
- **Versatile** - analyze data using multiple probes in a single well using a microscope, digital imager, or fluorescence plate reader
- **Flexible** - design kinetic or endpoint cell migration assays without the use of special instrumentation

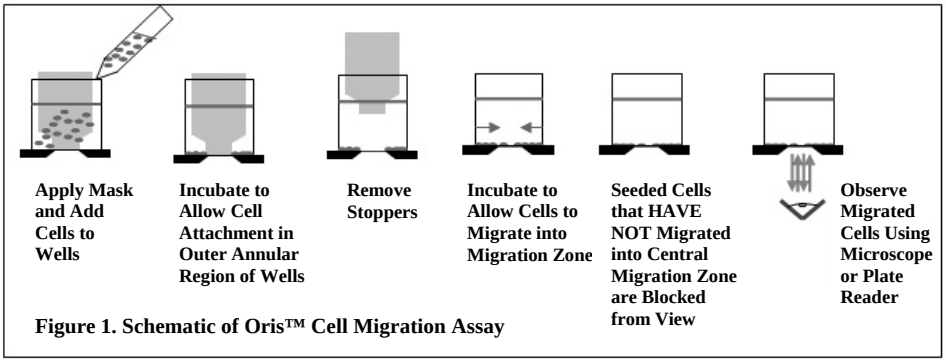


Figure 1. Schematic of Oris™ Cell Migration Assay

II. PRODUCT SPECIFICATIONS

Diameter of well	6.5 mm
Diameter of Stopper Space (Migration Zone)	2 mm
Suggested Media Volume per Well (populated with Stoppers)	100 µl
Suggested Media Volume per Well (after removal of Stoppers)	End-user determined
Effective Area of Outer Annular Region (seeding region) per Well	30.03 mm^2
Effective Area of Central Migration Zone per Well	3.14 mm^2

III. MATERIALS PROVIDED

- One (1) 96-well Plate with Oris™ Cell Seeding Stoppers
- One (1) Oris™ Migration Mask
- One (1) Oris™ Stopper Removal Tool

IV. MATERIALS REQUIRED

- Biological Cells
- Cell Culture Medium
- Sterile PBS
- Sterile Pipette Tips and Pipette or Multi-Channel Pipette
- Trypsin or Cell Scraper
- Inverted Microscope (optional)
- Fluorescence Microplate Reader (optional)
- Cell Labeling Fluorescent Agent (eg., CellTracker™ Green*, Calcein AM) - *required if performing assay readout via plate reader.* *a product of Molecular Probes/Invitrogen

V. CELL MIGRATION ASSAY PROTOCOL

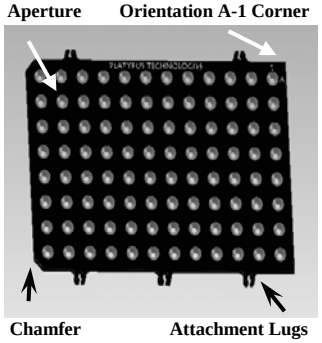


Figure 2. Features of Migration Mask

1. Apply the Oris™ Migration Mask to the bottom of the 96-well plate.

   First Time Users: In order to prevent splashing of well contents, familiarize yourself with the attachment and removal of the Migration Mask before any liquids are placed in the wells.

   - Orient the chamfered corners of the mask with those of the 96-well plate, ensuring that the A1 corner of the mask is aligned with the A1 well of the plate (see Figure 2).
   - Align the holes in the attachment lugs with the bosses on the bottom of the 96-well plate.
   - Gently press the mask until it is flush with the bottom of the 96-well plate.

NOTE: It may be necessary to wash the mask with ethanol to remove dust and debris. The mask may be applied at any point during the assay. For kinetic assays, it is often most convenient to apply the mask at the beginning of the assay before any liquids are placed in the well. For endpoint assays, using fixed and stained cells, it is often most convenient to apply the mask just before reading assay results.

2. If performing a kinetic analysis of cell migration, pre-stain with a fluorescent stain now.

3. Collect cells and prepare a suspension that is 10-fold greater in density than the optimal seeding concentration.

   First Time Users: The optimum seeding density of cells must be determined as an integral part of the design of the cell migration assay. Please see Appendix I for a discussion of this process.

4. Pipette 100µl of suspended cells into each test well through one of the side ports of the Cell Seeding Insert.

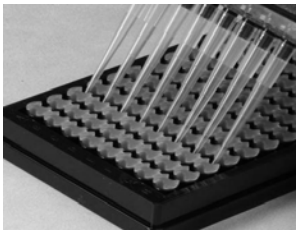


Figure 3.
Media is Added with Single or Multi-Channel Pipette

NOTE: For best results, add or extract media by placing the pipette tip along the wall of the well (see Figure 3). Care should be taken not to disturb the Cell Seeding Insert when introducing the pipette tip into the well. A gel loading tip may be useful.

5. Lightly tap the plate on your work surface to evenly distribute well contents (extreme tapping may result in splashing of well contents and lead to contamination).

6. Incubate the seeded plate containing the Oris™ Cell Seeding Stoppers in a humidified chamber (37 °C, 5% CO_2) for 10 to 16 hours (cell line dependent) to permit cell attachment.

7. Remove plate from incubator.

8. Designate several 'reference' wells (that will represent t=0) in which the stoppers will remain in place until results are read.

9. Using the Oris™ Stopper Removal Tool, remove all other stoppers (see Figure. 4).
   - Secure the 96-well plate by holding it firmly against the deck of your work space. Slide the tines of the removal tool under the backbone of the stopper strip, keeping the underside of the removal tool flush with the top surface of the plate.
   - Lift the removal tool ***vertically*** to gently remove the stopper.

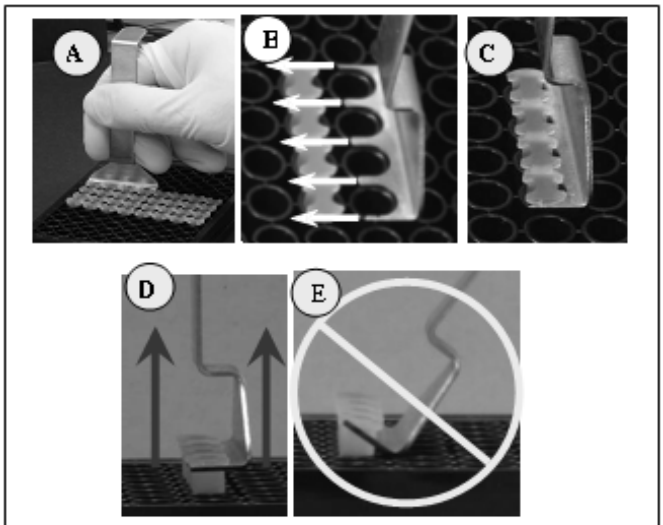


Figure 4. Removal of Stoppers
Panels A, B, and C) Position the Tines of the Removal Tool between the Stopper Tips, D) Lift Vertically, and E) Do NOT Pry Stoppers

NOTE: DO NOT use the removal tool as a lever to pry the stoppers from the well, as doing so may cause displacement of seeded cells.

10. Remove media and ***gently*** wash wells with 100µl PBS (or media) to remove unattached cells.

11. Add appropriate amount of fresh conditioned culture media to each well.

12. Incubate plate in a humidified chamber (37 °C, 5% CO_2) to permit cell migration. Incubation time will vary depending upon cell type and experimental design.

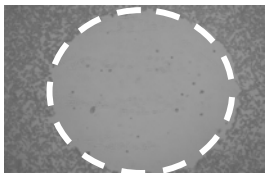
13. If performing an endpoint analysis of cell migration, apply stain.

VI. DATA ACQUISITION

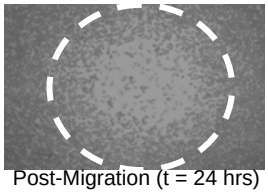
The readout of the Oris™ Cell Migration Assay can be conducted at any time, allowing the user to perform a kinetic assay or an endpoint assay. The Oris™ Cell Migration Assay is designed to be used with any commercially available stain or labeling technique. The readout can be performed by microscopic examination or by using a plate reader.

Microscopic Analysis

- Cell counting or image capture / analysis (using software, such as Image J freeware, available from NIH)
- Sample Data using a colorimetric stain is shown below. Wells were seeded with 50,000 HT1080 cells (i.e., $5x10^5$ cells/mL) and incubated for 4 hours. The stoppers were removed from test wells, but remained in place in the pre-migration reference wells until the time of the assay readout. The seeded plate was incubated in a humidified chamber for 24 hours to permit cell migration. Stoppers were removed from the reference wells and all cells were fixed and treated with Wright-Giemsa stain. Images were captured using bright field microscopy and then imported to Image J software for analysis using thresholding. The images below, captured without a migration mask in place, illustrate representative data from pre-migration (t=0 hrs) and post-migration (t=24 hrs) wells. The graph depicts the average pixel number in the migration zones for each condition.



Pre-Migration (t=0 hrs)



Post-Migration (t = 24 hrs)

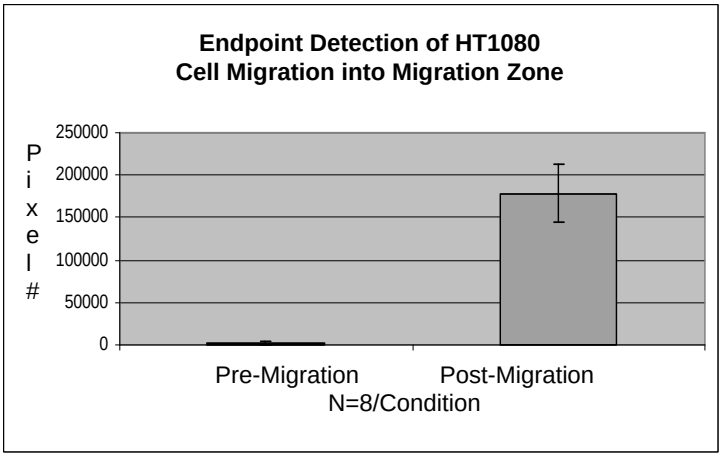
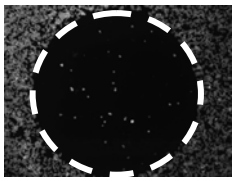


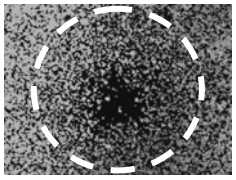
Plate Reader Analysis

- Setup on individual plate readers varies according to make and model. Consult your user manual for proper operation.
- The plate reader MUST be set to use the bottom probe read.
- Sample Data using a fluorescent stain is shown below. Wells were seeded with 50,000 HT1080 cells (i.e., $5x10^5$ cells/mL) and incubated for 4 hours. The stoppers were removed from test wells, but remained in place in the pre-migration reference wells until the time of the assay readout. All wells received CellTracker™ Green to fluorescently stain the cells. The seeded plate was incubated in a humidified chamber for 28 hours and at various time points the fluorescence signals in the migration zones were measured using a plate reader. The images below, captured without a migration mask in place, illustrate representative data from pre-migration (t=0 hrs) and post-migration (t = 21 hrs) wells. The graph depicts a real-time analysis of cell migration that was prepared by transposing the fluorescent signal into cell numbers by using a standard curve and a 5-Parameter Logistic-fit Equation.

Endpoint Detection

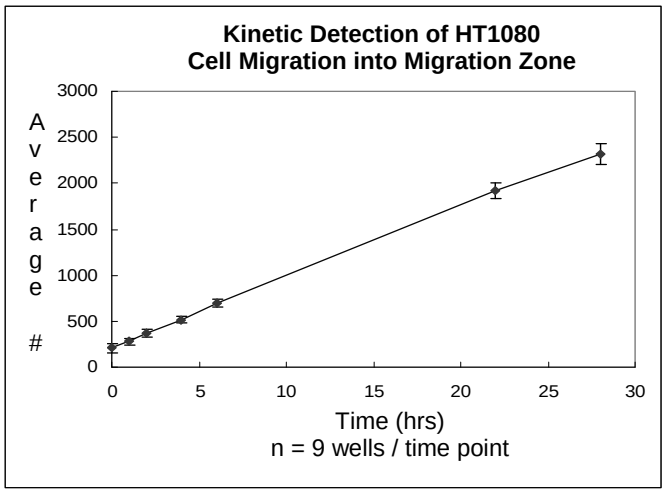


Pre-Migration (t= 0 hrs)



Post-Migration (t= 21 hrs)

Kinetic Detection



VII. ORDERING INFORMATION

Product No	Product Description	Package Size
CMA1.101	Oris Cell Migration Assay, 1-pack: One (1), 96-well plate with cell seeding stoppers One (1) Oris Migration Mask & One (1) Oris Stopper Removal Tool	1-pack
CMA5.101	Oris Cell Migration Assay, 5-pack: Five (5), 96-well plate with cell seeding stoppers One (1) Oris Migration Mask & One (1) Oris Stopper Removal Tool	5-pack
CMACC1.101	Oris Cell Migration Assay - Collagen Coated, 1-pack: One (1), 96-well plate (with Collagen I) with cell seeding stoppers One (1) Oris Migration Mask & One (1) Oris Stopper Removal Tool	1-pack
CMACC5.101	Oris Cell Migration Assay - Collagen Coated, 5-pack: Five (5), 96-well plate (with Collagen I) with cell seeding stoppers One (1) Oris Migration Mask & One (1) Oris Stopper Removal Tool	5-pack

To place an order, visit the Platypus Technologies website at: www.platypustech.com/order_main.html

For technical assistance, contact Technical Support at (866) 296-4455 or techsupport@platypustech.com

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APPENDIX I: Determining Optimal Cell Seeding Concentration

This appendix is intended to assist in determining the cell seeding density needed to achieve confluency of your cell line when using the Oris™ Cell Migration Assay. To that end, several dilutions of cell suspensions will be investigated.

NOTE: The Oris™ Migration Mask ***MUST*** be removed from the 96-well plate prior to the start of the following steps:

1. Collect cells by trypsinization or mechanical scraping and calculate total number of cells.
2. Pellet cells by centrifugation and resuspend to a final concentration of 500,000 cells/mL in culture media.
3. Seed a 100 µl portion of cells, at 2-fold serial dilutions in the 96-well plate starting at 50,000 cells/well (a suggested starting amount), as shown below. Keep in mind that the cell seeding area of the well with the stopper in place is ~ 0.3 cm^2 and based on the typical seeding density of your cells, you can infer the appropriate cell number for your first serial dilution.

Column	2	3	4
Cells / well	50,000	25,000	12,500
Number of wells	6	6	6

4. Incubate the plate in a humidified chamber (37 °C, 5% CO_2) for 16 hours with cell seeding stoppers in place.
5. Once the cells have attached, remove the Oris™ Cell Seeding Stoppers from each well (see Figure 4) and ***gently*** wash the wells with PBS to remove non-adhered cells.
   - Secure the 96-well plate by holding it firmly against the deck of your work space. Slide the tines of the removal tool under the backbone of the stopper strip, keeping the underside of the removal tool flush with the top surface of the plate.
   - Lift the removal tool ***vertically*** to gently remove the stopper. Do not use the removal tool as a lever to pry the stoppers from the well as doing so may cause displacement of the seeded cells.
6. Use a microscope to visually inspect the cells and determine the cell seeding concentration that yields a confluent layer.

NOTE: If you plan to obtain the results of the Oris™ Cell Migration Assay via colorimetric or microscopic analysis, you have successfully determined the optimal cell seeding concentration for your cell line. Proceed to Step 2 of the Cell Migration Assay Protocol. If you plan to obtain the results of the Oris™ Cell Migration Assay via a fluorescence plate reader, proceed with the following steps to optimize your plate reader settings.

7. The Oris™ Cell Migration Assay has been designed to work with all types of fluorescence stains and staining techniques. The precise method for staining cells with fluorescence stains varies according to the nature of the individual stain. Please consult the manufacturer of your fluorescence stain for specific considerations.

   First Time Users: For a guide to using Calcein AM, see below:
   a) Aspirate media from wells & wash wells with PBS or media.
   b) Add 100 µl of Calcein AM to each well at an appropriate concentration [for a fully-seeded 96-well plate, combine 5 µl of reconstituted Calcein AM (1mg/mL in dry DMSO) with 10 mL of serum-free media or 1x PBS].
   c) Incubate plate at 37 °C for 20 minutes.
   d) Remove plate from incubator.
   e) Aspirate staining solution.
   f) Fix cells, or to prevent drying, add 100 µl of 1x PBS to each well.

8. Apply the Oris™ Migration Mask to the plate.
9. Using the bottom probe of a fluorescence plate reader, obtain the total output from each well (adjust the gain settings to achieve optimal dynamic range). To determine optimal dynamic range, consider the following factors:
   a) The gain setting that permits detection of the lowest concentration of cells.
   b) The gain setting that permits discrimination between cell numbers at higher densities.

NOTE: When using a plate reader to analyze the Oris™ Cell Migration Assay, it is important to stain cells using a fluorescence reagent that uniformly stains cells. The use of a fluorescence probe that is affected by experimental conditions will increase variability of results and reduce correlation between fluorescence signal and cell migration. Fluorescence probes that are affected by experimental conditions could be utilized as counterstains for the study of factors and processes affecting cell migration.

You have successfully determined the optimal cell seeding concentration for your cell line. Proceed to Step 2 of the Cell Migration Assay Protocol.